Diacetyl and α-Acetolactate Overproduction by *Lactococcus lactis* subsp. *lactis* Biovar Diacetylactis Mutants That Are Deficient in α-Acetolactate Decarboxylase and Have a Low Lactate Dehydrogenase Activity

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*Lactococcus lactis* subsp. *lactis* biovar diacetylactis strains are utilized in several industrial processes for producing the flavoring compound diacetyl or its precursor α-acetolactate. Using random mutagenesis with nitrosoguanidine, we selected mutants that were deficient in α-acetolactate decarboxylase and had low lactate dehydrogenase activity. The mutants produced large amounts of α-acetolactate in anaerobic milk cultures but not in aerobic cultures, except when the medium was supplemented with catalase, yeast extract, or hemoglobin.

The flavoring compound diacetyl is an end product of citrate metabolism by certain lactic acid bacteria, such as *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. It arises from the chemical oxidative decarboxylation of α-acetolactate (4, 18), which can also be transformed to acetoin by α-acetolactate decarboxylase (ALDC) or by chemical nonoxidative decarboxylation. α-Acetolactate decarboxylation of *L. lactis* subsp. *lactis* biovar diacetylactis have the property of accumulating large amounts of α-acetolactate in the culture medium. Such strains are used in butter-making processes (19) and for the production of aroma additives with a high α-acetolactate (9) or diacetyl (8) content. The construction of an *L. lactis* strain having no ALDC and no lactate dehydrogenase (LDH) activity would be an interesting strategy for increasing diacetyl and α-acetolactate production. Indeed, a deficiency in LDH results in significant rerouting of the metabolic flux from lactose to acetoin synthesis (11). However, it appeared impossible to combine inactivation of the gene encoding ALDC with inactivation of the gene encoding LDH (6). We have observed that *L. lactis* mutants having various levels of attenuation of LDH activity can be generated by random mutagenesis (3). Our objectives were to induce such a mutation in ALDC-deficient strains and to determine if the resulting mutants are able to overproduce diacetyl and α-acetolactate.

**Obtaining mutants.** *L. lactis* subsp. *lactis* biovar diacetylactis Y8, MR3, and NO8 are ALDC-negative mutants that were selected from strains Y, MR, and NO (Laboratoire de Génie et Microbiologie des Procédés Alimentaires, Institut National de la Recherche Agronomique, Thiverval-Grignon, France), respectively, by a previously described method (13). This method is based on mutagenesis with *N*-methyl-∗N*-nitro-*N*-nitrosoguanidine followed by screening on agar plates. The three mutants were then subjected to a second mutagenesis procedure, in which the *N*-methyl-∗N*-nitro-*N*-nitrosoguanidine concentration was chosen to obtain 90% lethality. The cell suspension was inoculated onto LDHA-20 agar plates (7) in order to obtain approximately 1,000 colonies per agar plate. This medium enables the screening of large number of colonies for the presence of mutants having low LDH activity. However, some of the mutants detected on this medium probably do not display low LDH activity (7). The agar plates were incubated under aerobic conditions for 2 days at 30°C and then examined for the presence of brown colonies. Mutants forming brown colonies were evaluated for their ability to produce α-acetolactate in modified MR5 (5) medium, from which citrate was omitted. The cultures were incubated statically (partial anaerobiosis) for 24 h at 30°C, and α-acetolactate levels were determined as described by Mohr et al. (12).

The percentage of brown colonies and the percentage of α-acetolactate-producing mutants varied considerably among the strains (Table 1). The mean α-acetolactate concentrations were 3.01, 3.25, and 4.63 mM for mutants selected from strains Y8, MR3, and NO8, respectively. The reasons why most of the mutants that formed brown colonies were unable to produce α-acetolactate were not investigated. However, it is likely that several types of mutations, for example, those increasing the capacity for reducing 2,3,5-triphenyl tetrazolium, result in strains that form brown colonies on LDHA-20 agar but that do not overproduce α-acetolactate. No α-acetolactate-producing mutants could be detected among 200 colonies of strain NO8 that were white on LDHA-20 agar and that were selected at random. It is thus likely that there are no or very few α-acetolactate-producing mutants among white colonies.

**Stability of mutants.** Mutants were cultivated in M17 broth (17) for 24 h at 30°C. The cultures were then transferred daily as a 1% inoculum. Subcultures 1 and 10 were used to inoculate milk cultures (100 g/liter of reconstituted skim milk; sterilization for 10 min at 110°C), which were analyzed after 24 h at 30°C for their concentrations of α-acetolactate. Only one of the five mutants of strain Y8 had α-acetolactate production that did not vary significantly after 10 subcultures and was thus considered stable. The other four mutants produced less α-acetolactate, and the final pH of the corresponding cultures was lower. Five of the 14 mutants of strain MR3 and 2 of the 26 mutants of strain NO8 were stable.

**Enzymatic activities.** LDH and ALDC activities in cell extracts of the parent strains and of the eight mutants that were...
stable were measured by the procedures described by Boumerdassi et al. (3). None of the strains had detectable ALDC activity. The LDH activities of strains Y8, MR3, and NO8 were 21.8, 10.8, and 14.0 U/mg of protein, respectively. As expected, all of the mutants had lower LDH activity than the corresponding parent strains. The activity of the stable mutant of strain Y8 was 0.91 U/mg of protein, those of the mutants of strain MR3 varied between 0.01 and 7.07 U/mg of protein, and those of the two stable mutants of strain NO8 was 1.99 and 0.01 U/mg of protein.

Production of metabolites in milk cultures. Partial anaerobic cultures of strain MR3 and of three stable mutants selected from this strain were monitored for 24 h at 30°C (Fig. 1). Diacetyl and α-acetolactate levels were determined as described by Mohr et al. (12), and the sum of the levels of diacetyl plus acetoin was determined by the method of Westerfeld (20). The maximum concentration of α-acetolactate produced by the parent strain was 2.5 mM. It was obtained at 6 h of growth, a time which corresponded to citrate exhaustion (results not shown). The concentration of α-acetolactate decreased thereafter due to the instability of this compound, which can form acetoin or diacetyl. Mutants MR3-T1, MR3-T5, and MR3-T7 produced maximum α-acetolactate concentrations of 8.4, 13.5, and 10.0 mM, respectively. The maximum α-acetolactate concentration increased with time needed to reach that maximum. Diacetyl production was higher with the mutants than with the parent strain but was negligible compared with α-acetolactate production. Acetoin was the major degradation compound of α-acetolactate. In all of the cultures, the concentrations of diacetyl and acetoin increased without reaching a plateau, since α-acetolactate was not entirely degraded after 24 h.

Mutant MR3-T7 was also cultivated under aerobic conditions by incubating a 2-liter conical flask containing 500 ml of milk on a rotary shaker at 200 rpm. Surprisingly, the levels of acetoin and α-acetolactate were much lower than those in the partial anaerobic cultures (Fig. 2). Furthermore, the growth of the mutant was very poor, as the final absorbance of the culture, measured by the method described by Levata-Jovanovic and Sandine (10), was 0.26; that in partial anaerobic cultures was 4.56. Similar results were observed for the other stable mutants. This strong inhibition of growth by oxygen was not observed with the parent strain MR3 (results not shown), as already observed for the ALDC-negative mutant studied by Aymes et al. (2). The addition of 5 g of yeast extract (Fisher Scientific, Elancourt, France) that had been sterilized by filtration per liter, 70 U of catalase from bovine liver (Sigma, Saint-Quentin-Fallavier, France) per ml, or 0.2 g of Bacto Hemoglobin (Difco Laboratories, Detroit, Mich.) per liter resulted in a dramatic increase in diacetyl, acetoin, and α-acetolactate production by strain MR3-T7. The addition of these compounds also improved growth, as the final absorbances of the cultures were 4.28, 5.36, and 5.38 in the presence of yeast extract, catalase, and Bacto Hemoglobin, respectively.

Our findings show that L. lactis subsp. lactis biovar diaeclactis mutants that are deficient in ALDC and have low LDH activity are able to overproduce diacetyl and α-acetolactate, industrially significant compounds. The attenuation of LDH activity causes some of the metabolic flux to deviate toward the α-acetolactate synthase pathway, and the inactivation of ALDC causes the accumulation of α-acetolactate in the culture medium, to the detriment of acetoin. We did not investigate the reasons why most of the mutants were unstable, but we observed that after 10 subcultures, the unstable mutants formed mainly white colonies on LDHA-20 agar. They still produced some α-acetolactate, but their total levels of production of diacetyl, acetoin, and α-acetolactate were decreased. These results may reflect an instability of the mutations affecting LDH rather than those affecting ALDC. Furthermore, in

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<th>Percentage relative to colonies screened</th>
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<td>18.6</td>
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*a* Parent strains were deficient in ALDC.

*b* Screening was performed on LDHA-20 agar.

*c* Mutants that formed brown colonies on LDHA-20 agar and that produced more than 0.2 mM α-acetolactate when cultivated in modified MR5 medium, containing no citrate.

FIG. 1. Production of α-acetolactate, diacetyl, and acetoin in partial anaerobic cultures of strain MR3 (+) and mutants MR3-T1 ( ), MR3-T5 ( ), and MR3-T7 ( ).

FIG. 2. Production of α-acetolactate, diacetyl, and acetoin in partial anaerobic cultures of strain MR3 ( ), MR3-T1 ( ), MR3-T5 ( ), and MR3-T7 ( ).

TABLE 1. Screening of α-acetolactate-producing mutants of L. lactis subsp. lactis biovar diacetylactis

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other hypothesis is that the mutants may use hemoglobin to form a cytochrome-like respiratory system. As the formation of hydrogen peroxide by lactococci occurs mainly through the action of an NADH oxidase (1), the presence of a respiratory system would reduce the synthesis of hydrogen peroxide. A cytochrome-like respiratory system was detected in several lactococcal strains (15), and more evidence for respiration in lactococci in the presence of a heme derivative was provided recently by Sourice et al. (S. Sourice, M. Schaeffer, F. Violet, A. Gruss, and P. Duwart, Abstr. 6th Symp. Lactic Acid Bacteria Genet. Metab. Appl., abstr. G44, 1999).

Because of the negative effect of oxygen on the growth of the mutants, selection must be performed carefully. After mutagenesis, cells should be kept under anaerobic conditions or in the presence of yeast extract.

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REFERENCES